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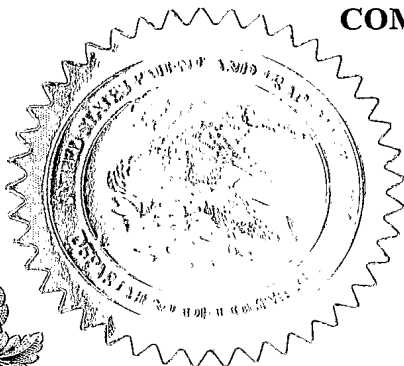
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Patent and Trademark Office, U.S. DEPARTMENT OF COMMERCE**PROVISIONAL APPLICATION COVER SHEET**

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53 (c).

Docket Number	4145-16	Type a plus sign (+) inside this box →	+
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INVENTOR(S)/APPLICANT(S)			
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TITLE OF THE INVENTION (280 characters)

METHOD FOR PRODUCING ANTIBODIES

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ENCLOSED APPLICATION PARTS (check all that apply)

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| <input checked="" type="checkbox"/> Specification | Number of Pages | 34 | <input type="checkbox"/> Applicant claims "small entity" status. |
| <input type="checkbox"/> Drawing(s) | Number of Sheets | | <input type="checkbox"/> "Small entity" statement attached. |
| | | | <input type="checkbox"/> Other (specify) _____ |

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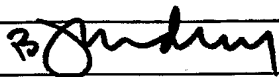
The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.



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Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,
SIGNATURE

DATE

August 12, 2004

TYPED or PRINTED NAME

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REGISTRATION NO.
(if appropriate)

36,663



Additional inventors are being named on separately numbered sheets attached hereto.

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U.S. PROVISIONAL PATENT APPLICATION

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Invention: METHOD FOR PRODUCING ANTIBODIES

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871741

LBP 1005 US02, Provisional Application

LONZA Biologics

Method for producing antibodies

Method for producing antibodies

5 The present invention relates to the field of biopharmaceuticals and to a method of producing recombinant antibodies in particular.

10 Monoclonal antibodies are becoming an ever more important class of pharmaceuticals. The elevated cost of manufacture entails a particular need to improve the primary yield of antibody in the cell culture supernatant to the utmost extent possible. Yield enhancement at the transcriptional level has been widely optimized, using strongest possible promoters and enhancer elements of mostly viral origin. Alike, as regards cell culture methodology and cell culture media, huge progress has been made for achieving high density growth,
15 high specific productivity and prolonged viability of cultured cells.

Therapeutic antibodies are mostly of the IgG-type, them being 150 KDa tetramers made up from two different sets of protein chains, a heavy (50 KDa) and a light chain (25 Kda). All chains made of multiple domains of the Ig-domain fold class. In particular the heavy
20 chains are not only glycosylated but the proper use of glycosylation sites and the correct composition of the carbohydrate moiety strongly affect quaternary structure. For instance, it has been found a decade ago that certain deposits of aggregated antibody correlated with a terminal galactosylation defect of the carbohydrate moiety of aggregated antibody. Further, there are intra- and interchain disulfide bonds contributing to assembly and
25 stabilizing the antibody structure under adverse extracellular conditions. In short, folding and assembly are equally crucial to efficient expression of antibody in recombinant cells.

Unfortunately, despite refined genetic expression technology, often a considerable portion of the light and heavy chains are not properly assembled. Partly, it is very difficult to
30 balance optimally light and heavy chain expression by promoter activity without having the one or other chain in excess. Further, there are assembly problems of the monomeric protein chains; even in the presence of 70% free IgG light chain, considerable amounts of monomeric IgG heavy chain may be detectable. Excess light chain may also be shedded to

the cell culture medium along with functional, fully assembled antibody. Simple chemical law of mass action does not apply here. Efforts to elevate selectively the expression level of the heavy chain have not proven successful. Gass et al., Trends Immunol. 15/1 (2004) suggest that in plasma cells, that is actively antibody secreting B-cells, the best achievable Mab titre is reached in the presence of excess light chain. Likely explanations are inefficient assembly and/or premature selective degradation targetting of the heavy chain; quality control and degradations timing are known to involve glucose tagging of the carbohydrate moieties. Protein domain assembly may be driven by affinity interactions, formation of disulfide bridges bringing domains into close proximity and/or the need to bury exposed more hydrophobic patches on the surface of individual domains; in the initial stage of assembly and folding, it is believed that such less patches are shielded by chaperone proteins. It is well-known nowadays that secretory glycoprotein is folded and assembled to higher order complexes in the inner, endoplasmic compartment (ER) of the cell structure only shortly after protein synthesis. The ER is the sole compartment to comprise specific auxilliary assembly factors along with quality control mechanism (Ellgaard et al., Quality control in the secretory pathway, Science. 1999 Dec 3;286:1882-8; Helenius et al., Intracellular functions of N-linked glycans, Science. 2001 Mar 23;291:2364-9.). Once the chance for successful assembly has passed, no assembly will further take place along further passage through the secretory pathway of the cell. In fact, some cell types such as CHO cells secrete unassembled chains of both type whereas others such as NSO cells retain selectively non-assembled chains of the IgG heavy type only and probably target them to degradative pathways.

Davis et al. (Effect of PDI overexpression on recombinant protein secretion in CHO cells, 2000, Biotechnol. Prog. 16:736-743) sought to increase secretion rate of a recombinant product protein comprising disulfide bridges in a widely employed industrial host cell system, namely CHO cells. Protein disulfide isomerase (PDI) was co-expressed with the product protein in addition to the endogenous expression level of PDI. Contrary to expectations, co-expression of PDI along with product protein led to reduced secretion rate and thus productivity; further, the product protein was retained inside the cells in the endoplasmic reticulum, colocalizing with PDI enzyme protein in the endoplasmic reticulum.

WO03/057897 teaches a method for expressing a recombinant protein comprising co-expression of chaperone proteins and small heat shock proteins. Those additional proteins are said to promote successful folding and assembly and thus the portion of correctly folded, most active product protein.

5

As a disadvantage, the co-expression of several auxilliary factors may decrease total expression rate of product protein and requires careful optimization of individual co-expression rates of such auxilliary factors. Different product protein might dependent to a varying degree on individual, only partially overlapping chaperone functions of which a multitude has become known to date, e.g. GroEL, GroES, DnaK, DnaJ, GrpE, ClpB, IbpA, IbpB... This whilst it is not desirable to co-express all of them at a time at the sole expense of product protein production rate.

10

15 Shu et al. (Proc. Natl. Acad. Science USA, 1993 Sept., 90: 7995-7999) describe construction of a single-chain immunoglobulin-like molecule that comprises the hinge and Fc portion (CH2 + CH3) of an IgG-type antibody, in contrast to the more routinely employed single chain antibodies made up from isolated variable domains. The design of the single-chain monomeric protein included a covalent linkage of the carboxyl terminus of the VH domain and the amino end of the VL domain through a (Gly-Gly-Gly-Gly-Ser)³ peptide linker. The carboxyl end of VL and the amino end of CH2 were joined through the hinge region. The single chain assembled into a dimeric molecule.

20

The idea behind this approach was that therapeutic effect will require the effector functions of whole antibodies having Fc constant portions. Furthermore, it is technically less demanding to express such whole fused antibody from a single host cell than by efficiently introducing and co-expressing two separate genes from heavy and light chain separately. Smaller fusion molecules such as commonly employed scFV antibody fragments suffer both from lack of effector function as well as from a lower affinity of antigen binding, despite identical variable portions. Further, their plasma half life is much shorter – whole antibodies having Fc portions are much less rapidly cleared from a patient's body.

30

The disadvantage being any such single fusion whole antibody gene approach is,

however, that the longer the residential time of such antibody in patient's body, the more relevant immunogenicity of any non-natural, extended portion of such artificial fusion protein becomes. The linker peptide represents such potentially immunogenic portion. It shall be the more immunogenic the longer it is; the composition of the linker peptide
 5 sequence may influence its immunogenicity. Of course, immunogenicity of the fusion protein and especially the linker portion of the protein might be diminished by further covalent modification such as PEGylation with polyethyleneglycol chains; such modification requires additional downstream processing of product protein and expensive clinical grade chemical reagents.

10 An approach analogous to Shu, but with duplicated variable domains and an accordingly longer peptide linker is described in Santos et al. (1999, Clinical Cancer Research 5:3118-3123).

The same disadvantages brought forward in relation to Shu et al. apply to this analogous
 15 approach, of course.

It is the object of the present invention to avoid the disadvantages of the prior art and to devise another, potentially less immunogenic method of expression of standard, tetrameric whole IgG antibody having Fc receptor activity and consisting of at least two different
 20 polypeptide chains. This object is solved by a method for producing an immunoglobuline having Fc receptor activity or complement activation activity which immunoglobuline when secreted from a vertebrate host cell comprises at least a first and a second polypeptide chain which two polypeptide chains are different, comprising the steps of

- 25 a. expressing in a vertebrate host cell having Golgi-only or late-Golgi-only resident furin family endoprotease activity an fusion polypeptide comprising a secretion targetting sequence directing the polypeptide to the secretory pathway and further comprising at least the first and second polypeptide sequences and at least one cleavage site for the said endoprotease activity
- 30 b. having the fusion polypeptide cleaved in the cells by the furin family endoprotease activity into the first and second polypeptide chains and

c. harvesting the secreted immunoglobuline.

An immunoglobuline according to the present invention has Fc-receptor activity or complement activation activity or both. Whereas complement activation is clearly defined in the art as to relate to induction of blood clotting (by possibly different pathways), Fc-receptor activity in the context of the present invention is to be understood as to the activation of cellular Fc receptors which trigger a cellular response, e.g. in the case of naturally occurring IgG or IgA triggered phagocytic or cytotoxic activities or e.g. in the case of release of mast cell granula upon triggering of cellular receptors by natural IgE class immunoglobulin. Similarly, amongst natural antibodies, both IgM and IgG class antibodies may trigger complement activation. It goes without saying that any such effector activities may vary amongst naturally occurring subclass antibodies, and accordingly may vary amongst the. In the context of the present invention, however, it is possible that Fc-receptor activity or complement activation effector domains are engineered into any given immunoglobulin structure by means of domain swapping, effectively transferring or adding the respective effector properties in such resulting immunoglobulin. The immunoglobulin may be a naturally occurring type of immunoglobuline, apart from its specific binding for a given antigen, or it may be an engineered, artificial type of immunoglobuline. This includes species-chimeric antibodies or CDR grafted antibodies, antibodies created by gene shuffling or site-directed engineering, antibodies chemically modified with PEG or radioisotope-chelating moieties or fusion proteins linking an immunoglobulin moiety having afore said activity to any other proteinaceous moiety such as another enzymatically active domain. The extend to which every activity is conferred by a given Immunoglobuline may vary. Both types of effector function are caused by the constant portion regions of the immunoglobuline heavy chain; for instance, the different human IgG subclasses vary in their relative efficacy to activate and amplify the steps of the complement cascade. In general, human IgG1 and IgG3 most effectively fix complement, IgG2 is less effective, and IgG4 does not activate complement. Assay formats to test for either of afore said activities are well-known to immunologists and other persons; suitable protocols may e.g. be found in standard immunochemistry lab manuals such as Harlow et al., Antibodies – a laboratory manual, Cold Spring Harbor Laboratory Press 1988. In naturally occurring immunoglobuline for

instance, light chains have a single constant region domain and heavy chains have several constant region domains. The naturally occurring antibody or immunoglobulin classes IgG and IgA naturally have three constant region domains, designated CH1, CH2 and CH3, and the IgM and IgE classes have four constant region domains. In contrast, e.g.

5 WO02/056910 devises artificial antibodies for human therapy that are devoid of the CH1 domain; such antibodies are encompassed by the notion of immunoglobulin according to the present invention as well.

10 In the context of the present invention, secretion is understood in the usual way as to mean release of matter from the outer cellular membrane to the surrounding, extracellular space. The method according to the present invention does not only allow

A host cell according to the present invention may be any vertebrate host cell line that can be, in contrast to primary cell lines, stably propagated in cell culture. Possible cell lines
15 are e.g. COS cells, NSO cells, CHO cells, HT1080 cells, PER-C6 cells, BHK cells, Sf-9 cells. In certain further possible embodiments of the present invention, it may also be possible to use plant cells including algal cells that may be grown in suspension cell culture which plant cells allow of having such antibody secreted from said plant cell under circumstances.

20 Preferably, the vertebrate host cells according to the present invention are mammalian cells, most preferably human cells such as e.g. HT1080 cells. More preferably, the human cells according to the present invention are selected from the group consisting of HT1080 cells and Per-C6 cells (Crucell B.V., Netherlands). Most preferably, the cells are HT1080
25 cells. For instance, HT1080 cells can be ordered as ATCC No. CCL-121 at the American Type Culture Collection, Manassas/VA, U.S.A.. HT1080 have been found to allow of enhanced product glycosylation when used in combination with glutamine synthetase selection marker system (WO 03/064630).

30 Preferably, the host cells as specified above are lymphoid cells, more preferably mammalian lymphoid cells, encompassing e.g. hybridoma, myeloma and trioma cells lines. Examples are e.g. non-secreting hybridoma such as SP2/0 and non-secreting

myeloma cells e.g. such as NSO cell line ECACC No. 85110503 (European Collection of Cell cultures, Centre for Applied microbiology, Salisbury/Wiltshire SP4 0JG; United Kingdom) from mouse or YB2/3.0 Ag20 (described in GB2070313) from rat. Myeloma cells such as NSO cells truly are B-lymphoid cell types, namely plasmacytoma cell lines, although being routinely addressed in the art as 'myelomas' (Barnes et al., Cytotechnology 32:109-123, 2000).

More preferably, the lymphoid vertebrate host cells are mammalian lymphoid cells, most preferably they are non-secreting rodent myeloma cells. As said below for the specific example of lymphoma proprotein convertase, such cell lines comprise suitable levels of endogenous furin endoprotease activity. Lymphoid cell lines are particularly preferred in combination with the preferred embodiment of furin endoprotease activity according to the present invention consisting only of an endogenous enzyme activity of the host cell.

It goes without saying that the level of furin endoprotease activity expressed in any of the above specified host cell types determines the extent to which the immunoglobuline molecule as finally secreted into the cell culture medium according to the present invention has indeed been cleaved by the endoprotease activity. Thus, a mixture of secreted immunoglobuline made up from uncleaved, fused and cleaved immunoglobuline polypeptides may be obtained. From such mixture, the fraction of cleaved, assembled immunoglobuline polypeptides can be obtained by further chromatographic separation techniques that are routine in the art. It might also be possible to remove uncleaved polypeptide by means of an affinity chromatography with an e.g. antibody-based stationary phase specifically recognizing and binding to the linker peptide.

Preferably, the immunoglobuline or Ig molecule comprises at least a hinge domain, a CH2 and a CH3 domain or functional variants thereof. Those domains form the essential Fc part e.g. in natural IgG. Detailed descriptions and definitions of these structural elements of an immunoglobuline are set forth in Amzel et al., Three-dimensional structure of immunoglobulins, *Ann. Rev. Biochem.* 48, 961-997 (1979); Davies et al., Structural basis of antibody function, *Ann. Rev. Immunol.* 1, 87-117 (1983); Hunkapiller et al., Diversity of immunoglobuline gene superfamily, *Adv. Immunol.* 44, 1-63 (1989). Said domains can be naturally occurring domains, artificially created chimeric versions of such domains or chimeric assemblies of such domains or versions engineered e.g. by site-directed

mutagenesis. In the past, chimeric, CDR grafted mouse human chimeric antibodies were often used; alike, potential glycosylation sites in the variable or CH1/CL domain portions were often eliminated by site directed mutagenesis. Of course, the extend of engineering of any part of the immunoglobuline according to the present invention may be often limited

5 by the need to avoid creating extended, strongly immunogenic motifs in engineered antibody, apart from the natural variability inherent to the complementarity determining regions. Apart from this, for the antigen-binding moiety upstream of the hinge portion that is conventionally coined the Fv portion of e.g. IgG type antibody, the only requirement according to the present invention is that such portion is made up from two distinct

10 polypeptide chains (when secreted) and has some antigen-binding property. It is possible that an immunoglobulin according to the present invention has increased antigen-binding valency achieved by multiplied variable domains arranged in a pearl-on-a-string fashion in its 'Fv' portion (similar to the suggestive drawing in Fig. 1 of Santos et al., Clinical Cancer Research, Vol. 5, 3118-3123, Oct. 1999, though the very antibody devised in Santos et al.

15 is based on the scFv concept employing very short interdomain linkers and thus probable has a different domain pairing pattern that shown in Fig. 1, making the large linker peptide shown in Fig. 1 a mandatory feature of Santos' antibody, in contrast to the present invention). Such 'Fv' portion, or what can be considered the equivalent of a naturally occurring Fv portion, may also be e.g. a shortened version that is devoid of the CH1 and CL

20 domain, or e.g. replace or enhances the CH1/CL domain pairing by a variable number of any other given, interfacing domain pair (e.g. VL/H domains or pairing domains unrelated to immunoglobuline but stemming from man, such as not being immunogenic). Essentially, an immunoglobulin according to the present invention is an antibody allowing of trigger Fc-receptor and/or complement activation activity and comprising said domain

25 elements; a functional variant of a known, natural domain equally complies with this requirement. The latter two activities appear both to located on or near the CH2 domain, but likely constitute different epitopes and are influenced by neighbouring domain elements and the tertiary and quaternary structure of the immunoglobulin. Proper domain interfacing as well as the feature of structurally important N-glycosylation and respective

30 N-glycan structure at Asn-297 (Roy Jefferis, Glycosylation of human IgG antibodies, Biopharm 2001, Advancstar Publication/U.S.A.; numbering from natural human IgG), spacing CH2 domains properly apart, may further influence said activities (Roy Jefferis, ibd.; Lund et al. Multiple Interactions of IgG with its core oligosaccharide can modulate

recognition by complement and human FcγRI and influence the synthesis of its oligosaccharide chains, *J. Immunol.* 164, 4178-4184 (2000)). Complement activation is initiated by binding of C1q, a subunit of the first component C1 in the blood clotting cascade, to an antigen-antibody complex. Even though the binding site for C1q is located in the CH2 domain of a natural antibody, the hinge region influences the ability of the antibody to activate the cascade – recombinant immunoglobulins lacking a hinge region are unable to activate complement. Studies have indicated that the hinge length and segmental flexibility correlate with complement activation; however, the correlation is not absolute. Human IgG3 with altered hinge regions that are as rigid as IgG4 still effectively activate the cascade. The hinge region is naturally found in IgG, IgA and IgD classes; as said already, it acts as a flexible spacer, allowing the Fab portion to move freely in space. In contrast to the constant regions, the naturally occurring hinge domains are structurally diverse, varying both in sequence and length amongst Immunoglobuline classes and subclasses. For example, three human IgG subclasses (IgG1, IgG2, IgG4) have hinge regions of 12-15 amino acids whilst the fourth, IgG3, comprises approximately 62 amino acids, including 21 proline residues and 11 cysteine residues. Crystallographic studies allowed of dividing the hinge region functionally into three different subregions: upper, core and lower hinge (Shin et al., *Immunological Rev.* 130:87 (1992)). The upper hinge includes amino acids from the carboxyl end of CH1 to the first residue in the hinge that restricts motion, generally the first cysteine residue that forms an interchain disulfide bond between the two heavy chains. The length of the upper hinge region correlates with the segmental flexibility of the antibody. The core hinge region contains the inter-heavy chain disulfide bridges, and the lower hinge region joins the amino terminal end of the CH2 domain and includes residues in CH2 (Shin et al., *supra*). The core hinge region of human IgG1 contains the sequence Cys-Pro-Pro-Cys which after formation of disulfide bonds results in a cyclic octa-peptide structure which may act as a pivot conferring flexibility. The hinge region may also comprise carbohydrate attachment sites, e.g. human IgA1 contains five carbohydrate sites within a 17 amino acid segment of the hinge region, conferring exceptional protease resistance to the hinge region.

Lack of the hinge region also affects the ability of human IgG immunoglobulins to bind Fc receptors on immune effector cells. Binding of an immunoglobulin to an Fc receptor facilitates antibody-dependent cellular cytotoxicity (ADCC), which is presumed to be an

important means to eliminate tumour cells. In the context of the present invention, Fc receptor activity is understood as ADCC activity as can be assayed with an appropriate target cell expressing the appropriate antigen by standard ^{51}Cr -release assay (see. e.g. Harlow et al., supra) or any more modern methods (e.g. Patel et al., J. Immunol Methods. 1995 Jul 17; 184(1): 29-38). In the context of the present invention, it is sufficient to find immunological effector cells (blood cells) from at least one species working in such assay as compared to an inactive, unrelated protein standard (e.g. serum albumin). Preferably, the immunological effector cells used to kill the target cells by means of ADCC in the assay are from human. — The human IgG Fc receptor family is divided into three groups Fc γ RI (CD64) which is capable of high affinity binding of human IgG, and Fc γ RII (CD 32) and Fc γ RIII (CD16), both of which are low affinity receptors. The molecular interaction between each of the three receptors and immunoglobulin has not been defined precisely but experiments indicate that residues in the hinge proximal region of the CH2 domain are important to the specificity of the interaction between the antibody and the Fc receptor (Lund et al., ibd.; Shields et al., 2001, J. Biol. Chem. 276:6591-6604). In addition, IgG1 myeloma protein and recombinant IgG3 chimeric antibodies that lack a hinge region are unable to bind Fc γ RI, purportedly because accessibility to CH2 is decreased (Shin et. al., Intern. Rev. Immunol. 10:177,178-179 81993).

Preferably, an immunoglobulin according to the present invention is of the IgG structural type and that the first polypeptide is an Ig-Light chain (L) comprising one VL and a CL domain, and that the second polypeptide is an Ig-Heavy Chain (H) comprising one VH, a CH1, a CH2 and a CH3 domain and a hinge domain. In this context, V stands for Variable domain comprising the complementarity determining region that forms the antigen binding pocket. H stands for heavy chain, L stands of course for light chain, C for Constant domain. More preferably, the CH1 to CH3 and hinge domain are of human IgG class or subclass or allotype.

Preferably, alone or in combination with any other preferred embodiment cited, the fusion polypeptide according to the present invention comprises the sequences of the first and second polypeptide separated by a linker. More preferably, the linker is positioned such as that the Light and Heavy Chain are separated by a linker and that the linker is cleaved off

from both Heavy and Light Chain by the furin family endoprotease activity. The linker according to the present invention is a linker peptide of course, linking heavy and light chain at the level of translation by allowing translation as a common fusion polypeptide from a single open reading frame. Examples of a suitable linker peptides linking heavy and light chain, or what can be said to correspond to them, can be found e.g. in Santos et al., *ibid.*, or Shu et al.. In general, a linker peptide should mainly comprise amino acids that promote an extended, fully solubilized conformation; small or possibly hydroxylated side chains such as found in glycine or serine would be first choice. In a preferred embodiment, the linker comprises one or several oligomers consisting of afore said amino acids glycine and serine. Equally preferred, alone or in combination, is that the linker is characterized by a content of >60% of all residues being glycine. Examples of such are (GGGGS)₆ or [AG₃S(G₄S)₂]₂. For a natural IgG-type immunoglobuline, preferably a linker's minimal length should be about 24-40 amino acids spanning the Light Chain's C-terminus to the Heavy Chain's N-terminus for example. Preferably, the linker comprises at least 20 amino acids. It goes without saying that any engineering by addition or deletion of domains of such natural antibody type would affect the minimal linker length required.

The furin endoprotease activity is an endoprotease activity naturally located exclusively in the late Golgi compartement or secretory vesicles further downstream in the secretory pathway. Usually, nascent newly synthesized furin-family endoproteases are proteolytically activated upon exit from the ER compartment only and gain full enzyme activity due to the distinctive pH and ionic strength (including particularly Ca²⁺ levels) features of the late Golgi or of the dense secretory vesicles in case of regulated secretion, as e.g. with proinsulin. Furin family endoproteases are Ca²⁺ dependent in general. The furin endoprotease family is a family of mammalian endoproteases sharing a common motif of catalytical residues forming the active site and further a domain motif termed the P or Homo B domain. Examples from mammalian cells are e.g. furin (PACE), the proinsulin convertases PC2 and PC3 or the furin homologue PACE 4. In baker's yeast, the mating or alpha-factor processing enzyme kex 2 is a homologue of mammalian furin endoproteases. A detailed description of said features and further references describing said two domain motif features are provided in the review Steiner et al., 1996, The role of prohormone convertases in insulin biosynthesis, *Diabetes & Metabolism* 22:94-104. The proteases of the furin endoprotease family cleave selectively at di- or multibasic sites in the

primary sequence. Most prohormones and neuroendocrine precursors, including proinsulin and proglucagon, that are processed and stored in dense core secretory vesicles, are cleaved at Lys-Arg (K-R↓) or Arg-Arg (R-R↓) sequences. Those proteases may require additional auxiliary protein factors for activation, in accordance with their role in regulated secretion only and accordingly are naturally expressed in a highly cell-type specific fashion, usually being expressed only in endocrine cell types. However, the precursors of many growth factors and various plasma proteins that are secreted via unregulated or constitutive pathways have more complex tetrabasic cleavage sites of the general Arg-X-Lys/Arg-Arg (R-X-K/R-R↓) type (Steiner et al., 1992, The new enzymology of precursor processing endoproteases, *J. Biol. Chem.* 267: 23435-23438; Yanagita et al., 1993, Processing of mutated proinsulin with tetrabasic cleavage sites to mature insulin reflects the expression of furin in non-endocrine cell lines; *Endocrinology* 133: 639-644; Molloy et al., 1994, Intracellular trafficking and activation of the furin proprotein convertase: localization to the Trans-Golgi network and recycling from the cell surface, *EMBO J.* 13: 18-33, Vey et al., 1994, Maturation of the trans-Golgi network protease furin: compartmentalization of propeptide removal substrate cleavage, and COOH-terminal truncation, *J. Cell. Biol.* 122: 1829-1842; Molloy et al., 1992, Human furin is a calcium-dependent serine endoprotease that recognizes the sequences Arg-X-X-Arg and efficiently cleaves anthrax toxin protective antigen, *J. Biol. Chem.* 267: 16396-16402; Rehemtulla et al., 1993, PACE4 is a member of the mammalian propeptidase family that has overlapping but not identical substrate specificity to PACE /Furin, *Biochemistry* 32: 11586-11590). The denotation 'K/R' is to be read as 'K or R' herein; kex 2 recognizes a dibasic cleavage site and is understood in the present context as a furin family endoprotease belonging to regulated secretion; yeast does not have constitutive secretion per se as do have higher eukaryotic cells, in particular mammalian cells. For the purpose of the present invention, lymphoma proprotein convertase (as described and further referenced in Loo et al., 1997, *J. Biological Chemistry*, Vol. 272, No. 43, pp. 27116-27123; the tetrabasic cleavage site motif for lymphoma proprotein convertase is further described in there) is understood as being another member of that furin endoprotease family and, having a tetrabasic cleavage site, is belonging to constitutive secretion. In the present context, such endoprotease belonging to constitutive secretion is also termed a 'constitutive' endoprotease activity.

Further, upon overexpression in CHO cells, lymphoma proprotein convertase was found

both to be still Golgi-only localized and not to be shedded to any extent into the supernatant, either. This protease activity is naturally present in lymphoid cell lines such as hybridoma and myeloma cells; myeloma cells truly are plasmacytoma, i.e. B-cell lineage derived cells. Further, non-secreting hybridoma such as SP2/0 or trioma cells may usually encompass such activity.

In the present context, a tetrabasic cleavage site or site motif recognized by the furin family endoproteases is defined as an contiguous tetrapeptide sequence comprising at least three basic residues selected from the group consisting of arginine and lysine. More preferably, the tetrapeptid sequence comprises even four basic residues selected from the group consisting of arginine and lysine. Such cleavage site motif is for instance described in Loo et al., 1997, supra.

According to the present invention, it is also possible that the furin family endoprotease activity is not the activity of a natural occurring furin family endoprotease but that of an artificially created, functional homologue. A functional homologue is defined as a modified furin family enzyme sequence preserving the characteristic features of 1. proteolytic activity in the environment of the late Golgi, 2. enzyme activation taking place after leaving the ER compartment and 3. Golgi-only or late Golgi-only localisation at least in one host cell species, and further having 4. preferably a basic cleavage site, more preferably a tetrabasic cleavage site motif. It is therefore conceivable to employ genetically engineered, in the above sense functional variants of known furin family endoprotease gene products in the present invention. Such variants can be generated e.g. by substitutions, deletions, insertions or truncations of the amino acid and its encoding DNA sequence, respectively. Methods for such are well known in the art and usually comprise specific site directed mutagenesis or generation of diversity by random mutagenesis of which is then followed by selecting desired variants by means of functional assays. Routine methods employed for mutagenesis may be e.g. exposure to alkylating agents or UV irradiation, error-prone PCR or related gene shuffling PCR techniques and are usually performed in microorganisms (Miller, J., Experiments in Molecular Genetics, Cold Spring Harbor Laboratory 1972; Ling et al., 1997, Approaches to DNA Mutagenesis, Analytical biochemistry 254, 157-178; Cadwell et al., 1992, Randomization of genes by PCR

mutagenesis in: PCR Methods, Cold Spring Harbor Laboratory Press 1992; Moore et al., 1997, Strategies for the in vitro evolution of protein function, J. Mol. Biol. 272, 336-347). Preferably, a functional homologue according to the above definition is at least 95% homologue at the amino acid level and is at least 70% homologue at the DNA level.

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In a further preferred embodiment of the present invention, the furin endoprotease activities are enzyme activities from vertebrate or more preferably mammalian furin endoprotease enzyme.

10 Further strongly preferred, either alone or in conjunction with the immediately preceding embodiment, is that the furin endoprotease activity is a constitutive endoprotease activity belonging to constitutive secretion pathway. Such endoprotease activity has an aforementioned tetrabasic cleavage site characteristically. It must be understood in the present context that antibodies are usually secreted by constitutive secretion; regulated
15 secretion requires sorting to a distinct subset of secretory vesicles. Furin endoproteases functioning in regulated secretion, e.g. insulin processing PC 2 and PC3, will only be active in those particular secretory vesicles; regulated secretion usually results in very large Ca influx. Sorting of protein to those distinct vesicles requires specific sorting signals, at least some of which are protein specific and poorly understood. Further, for the purpose of
20 the present invention, it goes without saying that the level e.g. of endogenous furin/PACE activity may vary. Chinese hamster ovary cells (CHO) have comparatively low endogenous furin activity; a heterologously expressed, furin-cleavable fusion protein may be secreted up to 30-50% by CHO cells in the uncleaved, native form. Overexpression of recombinant, CHO-derived furin enzyme may help to cleave such fusion protein
25 quantitatively. Despite overexpression, careful localization studies demonstrated that all furin activity was still properly Golgi-only localized. However, overexpression of Kex-2 activity in CHO cells, equipped with suitable secretion leader pre-sequence, has been reported to result in widespread expression of Kex-2 in the secretory pathway including the ER; unlike e.g. mammalian proinsulin convertases PC2 and PC3, Kex-2 proved to retain
30 considerable constitutive proteolytic activity under these conditions, resulting in early cleavage in the ER. However, according to the present invention, in case of recombinant host cells only properly Golgi-only or preferably late Golgi-only localized furin family endoprotease activity falls within the scope of the present invention. Localisation signals

for proper Golgi-only localisation of active endoprotease, ionic environment and autoprolytic activation in the ER contributing to the onset of enzymatic activity, are highly species dependent and may fail when protein is heterologously expressed in fairly distant species. Therefore in a further preferred embodiment of the present invention, the host cells according to the present invention are devoid of non-vertebrate, more preferably non-mammalian furin endoprotease activity stemming from native endoprotease enzyme such as e.g. Kex-2 of yeast. What is possible though, is to create properly localized, artificial hybrid enzymes assembled by a localization signal moiety and an active enzyme domain; since approach may not work out simply, a more elaborate combinatorial format for creating functional variants of furin endoproteases having proper Golgi-only or late Golgi-only localization properties should be chosen. Such approach is described for the similar task of glycosylation engineering by means of precisely localized, heterologous glycosyltransferases in Choi et al., Use of combinatorial genetic libraries to humanize N-linked glycosylation in the yeast *Pichia pastoris*, PNAS 2003, Vol. 100: 5022-5027 and WO 02/00879. WO 02/00879 describes requirements of adequate signal sequences for Golgi localization and pH optima requirements for protease domain activity in detail; these features are herewith incorporated to the present description. In a preferred embodiment of the present invention, suitable functional variants of the active enzymes of the furin endoprotease according to the present invention do not encompass such inter-species or chimeric protease enzymes obtained by combination of localization and protease domains of different species origin.

The furin family endoproteases according to the present invention generate proteinaceous fragments with C-terminal basic residues by virtue of their basic cleavage site motif, which basic residues are usually or mostly removed then by carboxypeptidase E, an exopeptidase. A host cell as defined above having suitable carboxypeptidase E activity is a further preferred embodiment of the present invention. 'Suitable carboxypeptidase E activity', in accordance with the present invention, is meant to be established in accordance with the present invention if at least 75%, more preferably at least 85%, more preferably at least 95% of protein cleaved by furin endoprotease activity and subsequently secreted is devoid of C-terminal basic (lysine or arginine) residue stemming from endoprotease cleavage site.

Preferably, the fusion polypeptide comprises at least two basic cleavage site motif recognized by a mammalian furin family endoprotease in the way that at least the linker separating the first and second polypeptide is linked to either polypeptide via a cleavage site, more preferably that the at least two cleavage site motifs are further recognized by a constitutively active furin family endoprotease and that the cleavage site motif is a tetrabasic furin family cleavage site motif accordingly. Given the fact that a given, single furin endoprotease may act on slightly varying cleavage site motifs (K/R content) with different kinetics, and that of course multiple different (and constitutive) furin endoprotease activities having different optimal cleavage site motifs and activity levels present in a single cell, it is possible according to the present embodiment that the afore said cleavage site motifs may be the same or different.

In a further preferred embodiment, the furin family endoprotease activity is an activity naturally present in the host cell line. That is, no recombinant engineering of furin endoprotease coding genes has taken place in such host cell line but only naturally occurring furin family endoprotease activities are present.

According to the present invention, preferably the host cell is devoid of detectable furin family endoprotease activity that is cleaving the fusion polypeptide in the endoplasmic reticulum, preferably is cleaving the fusion polypeptide at the basic cleavage site motif or motifs of the fusion polypeptide. The above employed wording 'Golgi-' or 'late-Golgi-only' imply this already. As is nowadays inferable as standard wisdom from cell biology textbooks, the secretory pathway is segmented into at least two very well distinguishable, well-defined compartments, the endoplasmic reticulum where protein folding, core glycosylation and protein assembly along with quality control of protein structure takes place, and the Golgi apparatus. Both morphological/microscopical and biochemical means allow of distinguishing in between these compartments; further, standard subcellular fractionation techniques employing gradient density centrifugation techniques can be employed as is routinely done in the art. Preferably, since the part of the Golgi that is most proximal to the ER, may in certain embodiments of the present invention allow of some recycling of membrane vesicles back to the ER, the furin family endoprotease activity according to the present invention is a late-Golgi only activity. As regards the molecular markers usually employed for distinguishing early (cis), medial and

late (trans) Golgi, reference is made to the standard textbooks. In the present context, late-Golgi as a term should be construed as not to exclude the presence of at least some amount of furin endoprotease activity being harbored in secretory vesicles in between the late Golgi and the outer cellular membrane.

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In a strongly preferred embodiment according to the present invention, the furin endoprotease activity comprises at least one recombinant furin endoprotease activity. The recombinant activity may be expressed from an episomal or chromosomal expression cassette; it may be heterologous expression of a furin family endoprotease homologue from
 10 another species or a functional variant thereof or expression of a host cell furin family endoprotease for achieving an elevated gene dosage. More preferably, such recombinant endoprotease is a constitutive furin family endoprotease activity or a functional variant thereof. Most preferably, such recombinant endoprotease is furin (PACE) endoprotease or lymphoma proprotein convertase (LPC) or a functional variant thereof.

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15 In such way, it is e.g possible to use CHO cells in the present invention for cleaving the fusion polypeptide carrying furin-specific cleavage site motifs quantitative. CHO cells have naturally low level furin endoprotease activity.

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In a further preferred embodiment, afore said recombinant such recombinant endoprotease
 20 is furin (PACE) endoprotease or lymphoma proprotein convertase (LPC) or a functional variant thereof and the host cell is a CHO host cell.

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Suitable media and culture methods for vertebrate and mammalian cell lines are well-known in the art, as described in US 5633162 for instance. Examples of standard cell
 25 culture media for laboratory flask or low density cell culture and being adapted to the needs of particular cell types are for instance: Roswell Park Memorial Institute (RPMI) 1640 medium (Morre, G., The Journal of the American Medical Association, 199, p.519 f. 1967), L-15 medium (Leibovitz, A. et al., Amer. J. of Hygiene, 78, 1p.173 ff, 1963), Dulbecco's modified Eagle's medium (DMEM), Eagle's minimal essential medium
 30 (MEM), Ham's F12 medium (Ham, R. et al., Proc. Natl. Acad. Sc.53, p288 ff. 1965) or Iscoves' modified DMEM lacking albumin, transferrin and lecithin (Iscoves et al., J. Exp. med. 1, p. 923 ff., 1978). It is known that such culture media can be supplemented with fetal bovine serume (FBS, also called FCS), the latter providing a natural source of a

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plethora of hormones and growth factors.

For high-density growth of the animal cells in an industrial fed-batch bioreactor according to the present invention, a high-density growth culture medium has to be employed.

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According to the present invention, a cell culture medium will be a high-density growth culture medium by definition if the culture medium allows for growth of animal cells up to or in excess of a density of *viable* cells of 10^5 - 10^6 cells/ml in a conventional fed-batch bioreactor system. Usually, such a medium will comprise 1-10 g/l Glucose or another
10 source of energy, the concentration of glucose being controlled at this level during fed-batch cultivation. Preferably, the medium will comprise at least 2 g/l Glucose, this concentration essentially being controlled during fed-batch fermentation. The medium is isotonic, namely being in the range of 270-320 mOsm/kg, preferably at 280-300 mOsm/kg. Individual preferences of certain cell types, e.g. lymphoid cells, for certain media are well-
15 known in the art, and are complexly correlated with the range, proportion and individual dosing of nutrients. Examples of a high-density growth media suited e.g. for hybridoma cell lines as compared to the standard media mentioned above are given in GB2 251 249 A and EP-435 911 A and e.g. for CHO cells are given in US 5,122,469; EP-435 911 and EP-229 809 do also describe suitable fed-batch regimes for cell culture, as shown in EP-229
20 809, the fed composition partly making up for elevated nutrient contents of inoculation media used in e.g. EP-435 911; such high-density growth media can be usually supplemented with nutrients such as all amino acids, energy sources such as glucose in the range given above, inorganic salts, vitamins, trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), buffers, the
25 four nucleosides or their corresponding nucleotides, antioxidants such as Glutathione (reduced), Vitamine C and other components such as important membrane lipids, e.g. cholesterol or phosphatidylcholine or lipid precursors, e.g. choline or inositol. A high-density medium will be enriched in most or all of these compounds, and will, except for the inorganic salts based on which the osmolarity of the essentially isotonic medium is regulated, comprise them in higher amounts (fortified) than the afore mentioned standard
30 media as can be incurred from GB2251 249 in comparison with RPMI 1640. Preferably, a high-density culture medium according to the present invention is balancedly fortified in that all amino acids except for Tryptophane are in excess of 75 mg/l culture medium.

Preferably, in conjunction with the general amino acid requirement, Glutamine and/or Asparagine are jointly in excess of 1 g/l, more preferably of 2 g/l of high-density culture medium. It goes without saying that the latter preferred embodiment is less suitable in case of a recombinant cell line transfected with a Glutamine synthetase (GS) vector

5 (Bebbington et al., 1992, High-level expression of a recombinant antibody from myeloma cells using a glutamine synthetase gene as an amplifiable selectable marker, Bio/Technology 10:169-175; Cockett et al., 1990, High level expression of tissue inhibitor of metalloproteinases in Chinese Hamster Ovary (CHO) cells using Glutamine synthetase gene amplification, Bio/Technology 8: 662-667). In comparison to the dihydrofolate
10 reductase (DHFR) system, the GS system offers a large time advantage during development because highly productive cell lines can often be created from the initial pool of transfectants thus avoiding the need for multiple rounds of selection in the presence of increasing concentrations of selective agent in order to achieve gene amplification (Brown et al., 1992, Process development for the production of recombinant antibodies using the
15 glutamine synthetase (GS) system, Cytotechnology 9:231-236). In such a GS cell line, an excess of e.g. glutamine stemming both from exogenous and endogenous source would lead to production of ammonia which is to be avoided.

It is also possible according to the present invention, to use a cell culture medium that is
20 devoid of fetal calf serum (FCS or FBS), which medium is consequently being termed 'serum-free'. Cells in serum-free medium generally require insulin and transferrin in a serum-free medium for optimal growth. Transferrin may at least partially be substituted by non-peptide siderophores such as tropolone as described in WO 94/02592. Most cell lines require one or more of synthetic growth factors (comprising recombinant polypeptides),
25 including e.g. epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin like growth factors I and II (IGFI, IGFII), etc.. Other classes of factors which may be necessary include: prostaglandins, transport and binding proteins (e.g. ceruloplasmin, high and low density lipoproteins, bovine serum albumin (BSA)), hormones, including steroid-
hormones, and fatty acids. Polypeptide factor testing is best done in a stepwise ashion
30 testing new polypeptide factors in the presence of those found to be growth stimulatory. There are several methodological approaches well-known in animal cell culture, an exemplary being described in the following. The initial step is to obtain conditions where the cells will survive and/or grow slowly for 3-6 days after transfer from serum-

supplemented culture medium. In most cell types, this is at least in part a function of inoculum density. Once the optimal hormone/growth factor/polypeptide supplement is found, the inoculum density required for survival will decrease. Further, it may also be possible to use modern protein-free media at least with certain host cell lines.

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Suitable bioreactors according to the present invention may be any culture system, for instance batch bioreactors such as e.g. airlift bioreactors or stirred bioreactors as routinely employed for high-density animal cell culture. Expediently, for high-density cell culture such bioreactor will be operated in a fed-batch mode. This definition includes continuous feed operation as well. Preferably, fed-batch bioreactors according to the present invention have a volumetric oxygen mass transfer coefficient K_{La} (as defined in Bailey, J. et al., Biochemical Engineering Fundamentals, McGraw-Hill, N.Y. 1986) of at least 6 h^{-1} , more preferably of at least 10 h^{-1} . Most preferably, a fed-batch bioreactor having said preferred oxygen mass transfer properties according to the present invention is an airlift bioreactor. Airlift bioreactors are well-known to the skilled person and the crucial parameters for reactor design have been well described (for review, see e.g. Chisti, M. et al., 1987, Airlift reactors, Chem. Eng. Commun. 60, 195-242; Koch, A. et al., 1987, Measurement and modeling of mass transport in airlift-loop reactors in relation to the reactor design, Chem. Ing. Tech. 59, 964-965).

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In another object of the present invention, the method of the present invention is employed in yeast based on yeast kex-2 activity and the corresponding dibasic cleavage site motif. Kex-2 is the natural furin family endoprotease activity of yeast and does not require any engineering of yeast host cell; it naturally generates active mating factors from prohormone sequences and helps directing them to secretion, whereas constitutive secretion in yeast is targetted to the vacuole. Of course, this requires that naturally occurring, incidental dibasic lysine/arginine motifs within the peptide sequence of the first and second polypeptide are to be avoided, eventually by genetic engineering of the immunoglobuline gene sequence. Further, expression of immunoglobuline usually requires suitable adaptation and engineering of yeast glycosylation, for rendering the resulting product immunologically acceptable for pharmaceutical dosing to humans. Glycosylation engineering in yeast, with the aim of humanized N-glycosylation of expressed, recombinant protein, is described in WO 02/00879 and Choi et al., supra. Since biopharmaceutical antibodies rarely harbor

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much further glycosylation sites than the conserved Fc-glycosylation site, a minimal biantennary core structure of the complex or oligomannose type of mammals would do. The work of Choi et al. have shown that such down-sizing of N-glycan structures in yeast is not refractory to yeast viability, contrary to general expectations.

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An at least biantennary Asn-GlcNAc₄ Man₃ moiety is to be understood as the empirical formula of the minimal N-glycan radical attached to asparagine (Asn). This definition encompasses of course mature, fully sialylated complex N-glycan structures that may be of from bi- up to tetraantennary and/or may also carry a further bisecting NacGlc residue. For possible constitutions, it is referred to the standard biochemistry textbooks and review articles.

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The description of possible and preferred embodiments in the foregoing apply likewise to this and all subsequent objects, where suitable. Namely it is again a further object of the present invention to devise a method for producing an immunoglobuline having Fc receptor activity and/or complement activation activity which immunoglobuline molecule when secreted from a vertebrate host cell comprises at least a first and a second polypeptide chain which two polypeptide chains are different, comprising the steps of firstly, expressing in a vertebrate host cell having Golgi-only or late-Golgi-only resident subtilisin/kexin family endoprotease activity an fusion polypeptide comprising a secretion targeting sequence directing the polypeptide to the secretory pathway and further comprising at least the first and second polypeptide sequences and at least one cleavage site for the said endoprotease activity, secondly having the fusion polypeptide cleaved in the cells by the furin family endoprotease activity into the first and second polypeptide chains and thirdly, harvesting the secreted immunoglobuline.

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Precursor endoproteases of the subtilisin/kexin family (Seidah et al., Ann. N.Y. Acad. Sci. 1999, 885:57-74, The subtilisin/kexin family of precursor convertases. Emphasis on PC1, PC2/7B2, POMC and the novel enzyme SKI-1.) have also been found to be exclusively Golgi localized (Seidah et al., Proc. Natl. Acad. Sci. USA, 1999 96:1321-1326, Mammalian subtilisin/kexin isozyme SKI-1: A widely expressed proprotein convertase with a unique cleavage specificity and cellular localization). Preferably, such kexin family

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endoprotease is human SKI-1, a mammalian homologue thereof or a functional variant thereof. Further similar to furin family endoproteases, the endoprotease is firstly translated as an inactive proprotein that is proteolytically activated upon exit from the endoplasmic reticulum; further, its activity is similiarly Ca^{2+} -dependent. In contrast, SKI-1 and kexin
5 endoproteases in general do not require strongly basic cleavage site motifs but do cleave in case of SKI-1 the sequence RGLT↓SL (arrow indicating the site of cleavage within the cleavage signal sequence motif). This has the advantage of leaving not leaving much basic, terminal residues left (except the Arginine in position 4), since endogenous cellular carboxypeptidase may in some cell types not efficiently remove all basic residues.

10 Terminal basic residues affect pI of protein and are believed to influence plasma half-life of antibody. It is to be recalled that in a preferred embodiment of the above object of the invention, an all-basic terminal tetrabasic cleavage site motif was preferred for reason of allowing removal of basic residues up to the last position 4-basic residue from the carboxyterminus and further for providing good exposure to aqueous solvent and hence
15 efficient cleavage. — By using kexin family proteases, carboxypeptidase trimming is not needed, however, the position-4 arginine is also definitely not to be removed, allowing of more uniform product protein in terms of pI but possibly allowing still of some impact on plasma half-life. It should also be mentiond that in contrast e.g. to recombinantly expressed furin, SKI-1 was found to be also located beside the Golgi in endosomal transport
20 vesicles on way to the outer cellular membrane and that further indeed some truncated, soluble version of natural, membrane bound SKI-1 enzyme was found to be steadily shed into the culture medium in 293 cells expressing the natural SKI-1 endoprotease gene (Seidah et al., supra). The reason for proteolytic truncation of the SKI-1 enzyme in vivo is unknown.

25 The afore fusion polypeptides comprising the polypeptid sequences of the mature antibody along with the respective furin or kexin family cleavage site motifs, are a further object of the present invention.

30 Examples

Expression of B72.3 antibody as a fusion polypeptide in CHO cells and CHO cells co-expressing recombinant furin endoprotease gene from CHO

5 The gene structure of mouse-human chimeric antibody cB72.3 is described in Whittle et al., Protein Eng. 1987, Dec. 1(6):499-505; the original hybridoma cell line B72.3 from which the recombinant chimeric antibody was constructed is obtainable from ATCC as ATCC No.HB-8108. For the purpose of the present experiment, an in-frame fusion is generated by linking the C-terminus of the light chain of cB72.3 via the sequence -Arg-Arg-Lys-Arg-(Gly-Gly-Gly-Gly-Ser)₆ -Arg-Arg-Lys-Arg- to the N-terminus of the heavy
10 chain of cB72.3, further eliminating the signal peptide coding sequence from the heavy chain coding sequence's N-terminus.

This in frame fusion is expressed in CHO-K1 cells exactly as described in Yanagita et al., (1993), Endocrinology 133:639-644, except for the fact that the cB72.3 in frame-fusion
15 substitutes for the proinsulin in the mammalian expression vector pcDL-Sralpha296 (Takebe et al., 1988, Mol.Cell.Biol. 8:466-472). The CHO-K1 cells (ATCC CCL-61) are optionally cultured as suspension adapted cells in another medium (Invitrogen CD-CHO or Hyclone HQSFM4) , with or without serum-supplementation.

20 Antibody is harvested from supernatant and tested for Fc-receptor activity by ADCC assay; about 50% of all fusion peptide expressed is cleaved to release the linker peptide. In CHO cells made recombinant with co-expressed furin endoprotease, up to 95% of the fusion peptide is cleaved and gives rise to functional antibody.

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Claims:

5 1. Method for producing an immunoglobuline having Fc receptor activity and/or complement activation activity which immunoglobuline molecule when secreted from a vertebrate host cell comprises at least a first and a second polypeptide chain which two polypeptide chains are different, comprising the steps of

10 a. expressing in a vertebrate host cell having Golgi-only or late-Golgi-only resident furin family endoprotease activity an fusion polypeptide comprising a secretion targetting sequence directing the polypeptide to the secretory pathway and further comprising at least the first and second polypeptide sequences and at least one cleavage site for the said
15 endoprotease activity

b. having the fusion polypeptide cleaved in the cells by the furin family endoprotease activity into the first and second polypeptide chains and

20 c. harvesting the secreted immunoglobuline.

2. Method according to claim 1, characterized in that the Ig molecule comprises at least a hinge domain, a CH2 and a CH3 domain.

25 3. Method according to claim 1, characterized in that the Ig molecule is a standard Ig molecule comprising a VL, a VH, a CL, a CH1, CH2 and CH3 domain and a hinge domain.

30 4. Method according to claim 3, characterized in that the first polypeptide is an Ig-Light Chain (L) and in that the second polypeptide is an Ig-Heavy Chain (H).

5. Method according to claim 1 or 4, characterized in that the fusion polypeptide comprises the sequences of the first and second polypeptide separated by a linker.

6. Method according to claim 4, characterized in that the Light and Heavy Chain are separated by a linker and that the linker is cleaved off from both Heavy and Light Chain by the furin family endoprotease activity.
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7. Method according to claim 6, characterized in that the fusion polypeptide comprises at least two cleavage sites.
8. Method according to claim 1, characterized in that the furin family endoprotease activity is an activity naturally present in that host cell line.
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9. Method according to claim 1, characterized in that the host cell is devoid of furin family endoprotease activity in the endoplasmic reticulum.
10. Method according to claim 9, characterized in that the host cell comprises at least one first recombinant furin family endoprotease activity and is devoid of activity from that first recombinant endoprotease in the endoplasmic reticulum.
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11. Method according to claim 10, characterized in that the first recombinant endoprotease is furin endoprotease or lymphoma proprotein convertase or a functional variant thereof.
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12. Method according to claim 10 or 11, characterized in the host cell is a CHO cell.
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13. Method according to claim 1, characterized in that the furin family endoprotease activity is a constitutive endoprotease activity.
14. Method according to claim 1, characterized in that the host cell line is a mammalian cell line.
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15. Method according to claim 14, characterized in that the at least one recombinant furin family endoprotease activity stems is a homologously expressed mammalian furin family endoprotease naturally present in that host cell line which further is an constitutive furin family endoprotease or furin family endoprotease belonging to constitutive secretion, in this way achieving an elevated expression level of the natural gene product in its native host cell environment.

16. Method according to claim 15, characterized in that the mammalian host cell line are CHO cells.

17. Method according to claims 1 or 7, characterized in that the cleavage sites is an contiguous tetrapeptide sequence comprising at least three basic residues selected from the group consisting of arginine and lysine.

18. Method according to claim 17, characterized in that the tetrapeptid sequence comprises four basic residues selected from the group consisting of arginine and lysine.

19. Method according to claim 7, characterized in that the linker is a non-naturally occurring amino acid sequence.

20. Method according to claim 19, characterized in that the linker comprises at least 20 amino acids.

21. Method according to claim 20, characterized in the linker comprises one or several oligomers consisting of only glycine and either serine, threonine or both.

22. Method according to claim 21, characterized in that the linker consists of one or several oligomers consisting of only glycine and either serine, threonine or both.

23. Method according to claim 22, characterized in that the linker comprises at least >60% glycine residues.

24. Method for producing an immunoglobuline molecule having Fc receptor activity and/or complement activation activity which immunoglobuline molecule comprises multiple copies each of at least a first and a second polypeptide chain when
5 secreted from a host cell and which two polypeptide chains are different, comprising the steps of:

- 10 a. expressing in a vertebrate host cell having Golgi or late-Golgi-resident furin family endoprotease activity an fusion polypeptide comprising a secretion targetting sequence directing the polypeptide to the secretory pathway and further comprising at least the first and second polypeptide sequences and at least one cleavage site for the said endoprotease activity
- 15 b. having the fusion polypeptide cleaved in the cells by the furin family endoprotease activity into the first and second polypeptide chains and
- c. harvesting the secreted, functional immunoglobuline molecule.

20 25. Method for producing an immunoglobuline having Fc receptor activity and/or complement activation activity which immunoglobuline molecule when secreted from a host cell comprises at least a first and a second polypeptide chain which two polypeptide chains are different, comprising the steps of

- 25 a. expressing in a yeast host cell having kex2 endoprotease activity and humanized N-glycosylation an fusion polypeptide comprising a secretion targetting sequence directing the polypeptide to the secretory pathway and further comprising at least the first and second polypeptide sequences and at least one cleavage site for the said kex-2 endoprotease activity
- 30 b. having the fusion polypeptide cleaved in the cells by the kex-2 endoprotease activity into the first and second polypeptide chains and

c. harvesting the secreted immunoglobuline molecule

26. Method according to claim 47, characterized in that the yeast host cell is *Saccharomyces cerevisiae* or a methylotropic yeast, preferably a *Pichia* species.

27. Method according to claim 48, characterized in that the yeast host cell is *Saccharomyces cerevisiae*.

28. Method according to claim 47, characterized in that the meaning of humanized N-glycosylation is that the yeast host cell is devoid of outer chain glycosylation and preferably is capable at least of generating biantennary oligo-mannose N-glycan structures and/or complex N-glycan core structures comprising at least an biantennary Asn-GlcNAc₄Man₃ moiety.

29. Method according to claim 28, characterized in that the N-glycan comprises an bisecting N-acetyl-glucosamine residue enhancing Fc-receptor activity of the glycosylated antibody thus produced from the host cell.

30. Host cell for producing an immunoglobuline having Fc receptor activity and/or complement activation activity which said immunoglobuline molecule when secreted from the host cell comprises at least a first and a second polypeptide chain which two polypeptide chains are different, said host cells expressing Golgi-only or late Golgi only resident furin family endoprotease activity and further expressing a fusion polypeptide comprising a secretion targetting sequence directing the polypeptide to the endoplasmic reticulum and hence the secretory pathway for secretion to the extracellular space, which fusion polypeptide comprises at least the first and second polypeptide sequences and at least one cleavage site for the said endoprotease activity.

31. Host cell according to claim 30, characterized in that the immunoglobuline comprises multiple copies each of at the least the first and second polypeptide chain when secreted from the host cells.

32. Host cell according to claim 31, characterized in that the host cell is a fungal host cell, preferably a yeast host cell, having kex2 endoprotease activity and humanized N-glycosylation and wherein the at least one cleavage site is cleavable by the said kex-2 endoprotease activity

33. Host cell according to claim 32, characterized in that the kex-2 endoprotease activity is a natural kex-2 endoprotease activity present in that host cell.

34. Host cell according to claim 33, characterized in that the natural kex-2 endoprotease activity is vector-borne in said host cell.

35. Host cell according to claim 30 or 31, characterized in that the host cell is a vertebrate host cell.

36. Host cell according to claim 36, characterized in that the host cells is a mammalian host cell.

37. Host cell according to any of the preceding claims, characterized in that the Ig molecule comprises at least a hinge domain, a CH2 and a CH3 domain.

38. Host cell according to claim 38, characterized in that the Ig molecule is a standard Ig molecule comprising a VL, a VH, a CL, a CH1, CH2 and CH3 domain and a hinge domain.

39. Host cell according to claim 41, characterized in that the first polypeptide is an Ig-Light Chain (L) and in that the second polypeptide is an Ig-Heavy Chain (H).

40. Host cell according to any of the preceding claims, characterized in that the fusion polypeptide comprises the sequences of the first and second polypeptide separated by a linker.

41. Host cell according to claim 40, characterized in that the Light and Heavy Chain are separated by a linker by means of at least two cleavage sites and that the linker is cleavable off from both Heavy and Light Chain by the furin family endoprotease activity.

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42. Host cell according to claim 30, characterized in that the furin family endoprotease activity is an activity naturally present in that host cell line.

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43. Host cell according to claim 36, characterized in that the host cell is devoid of furin family endoprotease activity in the endoplasmic reticulum.

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44. Host cell according to claim 36, characterized in that the host cell comprises at least one first recombinant furin family endoprotease activity and is devoid of activity from that first recombinant endoprotease in the endoplasmic reticulum.

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45. Host cell according to claim 45, characterized in that the first recombinant endoprotease is furin endoprotease or lymphoma proprotein convertase or a functional variant thereof.

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46. Host cell according to claim 37 or 45, characterized in that the host cell is a CHO cell.

47. Host cell according to claim 36 or 37, characterized in that the furin family endoprotease activity is a constitutive endoprotease activity.

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49. Host cell according to claim 45, characterized in that the at least one recombinant furin family endoprotease activity stems is a homologously expressed mammalian furin family endoprotease naturally present in that host cell line which further is an constitutive furin family endoprotease or furin family endoprotease belonging to

constitutive secretion, in this way achieving an elevated expression level of the natural gene product in its native host cell environment.

50. Host cell according to any of the preceding claims, characterized in that the
5 cleavage sites is an contiguous tetrapeptide sequence comprising at least three basic residues selected from the group consisting of arginine and lysine.

51. Host cell according to claim 50, characterized in that the tetrapeptid sequence
10 comprises four basic residues selected from the group consisting of arginine and lysine. -

52. Host cell according to claim 50, characterized in that the linker is a non-naturally
15 occurring amino acid sequence.

53. Host cell according to claim 50, characterized in that the linker comprises at least
20 amino acids.

54. Host cell according to claim 50, characterized in the linker comprises one or
20 several oligomers consisting of only glycine and either serine, threonine or both.

55. Host cell according to claim 54, characterized in that the linker consists of one or
several oligomers consisting of only glycine and either serine, threonine or both.

56 Host cell according to claim 55, characterized in that the linker comprises at least
25 >60% glycine residues.

57 Method for producing an immunoglobuline having Fc receptor activity and/or
30 complement activation activity which immunoglobuline molecule when secreted from a vertebrate host cell comprises at least a first and a second polypeptide chain which two polypeptide chains are different, comprising the steps of

a. expressing in a vertebrate host cell having Golgi-only or late-Golgi-only
35 resident subtilisin/kexin family endoprotease activity an fusion polypeptide comprising a secretion targetting sequence directing the polypeptide to the

secretory pathway and further comprising at least the first and second polypeptide sequences and at least one cleavage site for the said endoprotease activity

- 5 b. having the fusion polypeptide cleaved in the cells by the subtilisin/kexin family endoprotease activity into the first and second polypeptide chains and
- 10 c. harvesting the secreted immunoglobuline.

Abstract

A novel method for expression of immunoglobulines is devised.

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